Retinoids Upregulate Phosphoenolpyruvate Carboxykinase and Glyceroneogenesis in Human and Rodent Adipocytes¹,²

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Abstract

Glyceroneogenesis is an important metabolic pathway for fatty acid reesterification in adipose tissue, thereby reducing fatty acid release. Glyceroneogenesis and cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C), which is the key enzyme in this pathway, are both regulated by a series of hormones and nutrients, among which all-trans retinoic acid (all-trans RA) is a transcriptional inducer of the PEPCK-C gene (Pck1). All-trans RA binds to the retinoic acid receptor (RAR) and activates it, whereas its stereoisomer 9-cis retinoic acid (9-cis RA) is a ligand for the 9-cis RA receptor (RXR). Three RXR-binding elements [retinoic acid response element (RARE)1/PCK1, RARE2, and RARE3/PCK2] were previously located in the promoter of Pck1. Using 3T3-F442A adipocytes, we demonstrated that Pck1 expression was 10-fold more sensitive to 9-cis RA (EC₅₀: 10 nmol/L) than to all-trans RA. We then analyzed the respective involvement of RARE1/PCK1, RARE2, and RARE3/PCK2 in the response of Pck1 to 9-cis RA and all-trans RA in adipocytes. The response to 9-cis RA mainly involved the RARE1/PCK1 element, whereas RARE2 was mainly responsive to all-trans RA. In contrast, the full response to both RA isoforms involved these 2 elements and included RARE3/PCK2 as well. Furthermore, 9-cis RA, but not all-trans RA, selectively induced PCK1 in ex-vivo-treated human adipose tissue explants, with a concomitant induction of glyceroneogenesis monitored by [1-¹⁴C]-pyruvate incorporation into neutral lipids. The concomitant 9-cis RA-induced reduction in fatty acid output indicates an important role for this RA stereoisomer in lipid homeostasis through stimulation of PEPCK-C and glyceroneogenesis in adipose tissue. J. Nutr. 138: 1004–1009, 2008.

Introduction

During fasting, white adipose tissue (WAT)⁵ is the key provider of nonesterified fatty acids (NEFA) to the blood and this is the consequence of lipolysis from stored triacylglycerol. A large portion of lipolytically derived NEFA (30–70%) are reesterified within adipocytes (1,2). This reesterification process requires glycerol-3-phosphate synthesis, which, under fasting conditions, originates from lactate or pyruvate by a pathway named glyceroneogenesis, because glycerol phosphorylation is low in WAT and glycolysis is much reduced (3). In obesity and in type 2 diabetes, regulation of WAT glyceroneogenesis is altered in both rodents and humans (4,5). Accordingly, antidiabetic thiazolidinediones (TZD) acutely and selectively induce WAT glyceroneogenesis in rodents and humans, thereby reducing NEFA release and lipotoxicity (4–6). The cytosolic isofrom of phosphoenolpyruvate carboxykinase (PEPCK-C) (3) is the key enzyme of this metabolic pathway. In addition to TZD, a series of nutrients and hormones modulate glyceroneogenesis by means of alterations in PEPCK-C gene (Pck1) regulation and PEPCK-C synthesis. The vitamin-A physiological derivative all-trans retinoic acid (all-trans RA), unsaturated fatty acids, and β-agonists acutely stimulate Pck1 transcription in adipocytes, whereas glucocorticoids and glucose are inhibitors (7–9).

PEPCK-C is also expressed in the liver where it is primarily involved in gluconeogenesis. The regulation of Pck1 expression has been extensively studied and there are major differences between liver and adipose tissue. For instance, glucocorticoids induce Pck1 transcription in liver while downregulating the
same gene in WAT (7–9). TZD stimulate Pck1 transcription in adipocytes but are ineffective in hepatocytes or hepatoma cells (9, 10). This could be due to the particularly weak expression in the latter cells of PPARγ, for which TZD are specific ligands. However, although the liver-predominant isoform of PPAR (PPARα) is expressed at high levels in hepatoma cells, selective ligands for PPARα cannot induce Pck1 expression in these cells (11). These examples indicate that Pck1 is differentially regulated in cell types that have divergent primary metabolic functions.

The mechanism by which all-trans RA stimulates Pck1 transcription has been extensively studied in hepatoma cells. Lucas et al. (12) and Scott et al. (13) originally demonstrated the existence of 2 retinoic acid response elements in the 5′-flanking region of the gene. Retinoic acid response element (RARE)/PCK1, the first element that was discovered, has a direct repeat 1 (DR1) structure located between −451 and −439 bp relative to the transcription start site of the rat gene. The other (RARE2) is a DR5-type element and is located between −337 and −321 bp. Both elements bind retinoic acid receptor (RAR)/RXR heterodimers and each mediates >50% of RA induction (12, 13). Shin et al. (14) created transgenic mice that express bovine growth hormone under control of the rat Pck1 promoter and then made them deficient. These studies demonstrated that RA was required for the proper expression and regulation of Pck1 and that the 2 elements apparently mediated these effects (14, 15). RXR also binds a second DR1-type element (RARE3/PCK2) located between −999 and −987 bp of the rat Pck1. Originally described by Tontonoz et al. (16) as a PPAR/RXR binding site. In vivo experiments in transgenic mice showed that RARE3/PCK2 was also able to bind PPARα (17). In addition, a recent report by Schug et al. (18) demonstrated that all-trans RA could activate PPARβ/δ in keratinocytes. Because PPARβ/δ is expressed in adipocytes, one could also assume that all-trans RA could induce Pck1 by PPARβ/δ/RXR binding to RARE3/PCK2. However, neither binding of PPARβ/δ/RXR to RARE3/PCK2 nor the involvement of the latter in the RA response of Pck1 in adipocytes is known.

The RARE3/PCK2 element was shown to confer some adipocyte-selective expression of Pck1 both in cultured cells and in transgenic mice (16, 19). Further studies by our group established that RARE3/PCK2 was the single element of the rat promoter that mediated TZD induction of Pck1 (11). This result was surprising because TZD are specific PPARγ ligands and activators and because the other DR1-like element in Pck1, RARE1/PCK1, is also able to bind a PPARγ/RXRα heterodimer (19). The nonresponsiveness of RARE1/PCK1 to TZD addresses the questions of which transcription factor primarily binds to this element in adipocytes and what this element is actually responsible for. Although we demonstrated earlier that all-trans RA induced transcription of Pck1 in adipocytes, we did not determine the response element(s) involved in this effect (20). Similarly, the relative importance of the 3 RXR binding sites, RARE1/PCK1, RARE2, and RARE3/PCK2, in the response of Pck1 to 9-cis RA and all-trans RA has not been addressed. Finally, the relevance of all these effects in humans needs to be explored, because retinoids have been shown to ameliorate glycemic control in rodent models of type 2 diabetes (21, 22).

The aims of this work were to: 1) study retinoid regulation of PEPCCK-C expression in rodent adipocytes and human WAT; 2) gain further insights into the molecular mechanisms involved in retinoid action; and 3) analyze the functional relevance of this regulation on lipid metabolism.

### Materials and Methods

**Materials.** DMEM was from Invitrogen. [α-32P]-dATP, [14C]-chloramphenicol, Hybond-N⁺ blotting membranes, and X-ray films were from Amersham. Random priming kit and Quick Hyb hybridization solution were from Stratagene. The Rous sarcoma virus promoter driving the nlsLaCZ gene was a gift from the “Laboratoire de Thérapie Génique” (Centre Hospitalier Universitaire de Nantes, France). RA, fetal bovine serum, essentially fatty acid-free bovine serum albumin (BSA), and all other products were purchased from Sigma.

**Cell culture and treatment.** 3T3-F442A cells and PEPCCK-C-CAT transient transfectants were cultured as previously described (11). Experiments were carried out on adipocytes (8 d after confluence) that had been maintained in serum-free, hormone-free medium for the previous 24 h.

**Animal studies.** Six-week-old male Sprague-Dawley rats were obtained from Janvier Laboratories. They were allowed to acclimate for 2 wk on a 12-h-light/dark cycle at constant room temperature before being killed by CO₂ asphyxiation. Rats consumed a standard balanced diet (60% carbohydrates, 16% proteins, 3% lipids, and 5% vitamins and minerals, including 6600 UI vitamin A (retinol), 900 UI cholecalciferol, 30 UI vitamin E (α-tocopherol), and 17 mg/kg oligoelements) from Safe and tap water ad libitum. The protocol for the animal studies was conducted according to the French Guidelines for the Care and use of Experimental Animals.

**Culture of human adipose tissue explants.** Explants of subcutaneous WAT were obtained from 8 healthy women undergoing elective surgery. They were aged 45.7 ± 4.4 yr (mean ± SD) and had BMI of 30.3 ± 1.4 kg/m². None of the subjects suffered from known metabolic or malignant diseases nor were they taking medications known to alter adipocyte metabolism. The study was performed according to the Declaration of Helsinki. All the patients gave their informed written consent. Subcutaneous WAT was then cultured as previously described (5).

**RNA extraction and analysis.** Total RNA was extracted from cultured cells using the Chomczynski and Sacchi method (23) and from human and rat WAT explants using the RNeasy total RNA kit from Qiagen. For 3T3-F442A cells, total RNA was subjected to Northern blotting as described (11). The GAPDH cDNA probe was used as a control.

For human WAT, total RNA (1.25 μg) was analyzed by real-time RT-PCR as described (5). Quantification of mRNA was carried out by comparison of the number of cycles required to reach reference and target threshold values with the Δ-ΔCT method.

**Analysis of enzyme activity and quantification.** 3T3-F442A cells were homogenized in ice-cold 10 mmol/L Tris-HCl buffer, pH 7.4, containing 250 mmol/L sucrose, 1 mmol/L EDTA, and 1 mmol/L dithiothreitol in the presence of 0.01% Triton X-100. After centrifugation of the homogenate at 3000 × g; 10 min, the supernatant was centrifuged at 25,000 × g; 45 min. Fractions were stored at −80°C until used. PEPCCK-C activity and protein amount were assayed as described (5). Protein concentrations were determined using the Bradford method using BSA as the standard.

**Plasmid constructs, transient transfections, and determination of CAT activity.** Plasmid constructs containing −2086 to +69 bp of the rat PEPCCK-C gene fused to the CAT gene with mutated RARE1/PCK1, RARE2, and/or RARE3/PCK2 elements have been previously described (11–13). Plasmid constructs including either mutated elements (PCK2-RARE1/PCK1, PCK2-RARE2, or RARE1-RARE2) or the 3 mutated elements were carried out.

Gene transfer experiments were carried out 8-d postconfluent 3T3-F442A differentiated adipocytes using the previously described polyethyleneimine adenofection method using Rous sarcoma virus promoter driving the nlsLaCZ gene (24). Seven hours later, the medium was discarded and fresh medium containing effectors was added for an additional 16 h. Cell homogenates and CAT activity have been described elsewhere (20).
Metabolic studies. WAT (200 mg) was cut into 20-mg pieces and incubated in 1.5 mL DMEM containing 3% (wt/v) FA-free BSA and 5 mmol/L pyruvate. [1-14C] pyruvate (20 MBq/L) was used at an isotopic dilution of 1/250. Two hours later, fragments were rinsed and lipid extraction was carried out as described [5]. The fatty acids half micro test from Roche was used to monitor NEFA release in the culture medium.

Image quantification and data analysis. Quantitative results were obtained by densitometry. The nonparametric Mann-Whitney U test for pairwise comparisons, namely between treated and control samples at each time point or between 9-cis RA and all-trans RA, was performed due to the small number \((n < 10)\) of experiments. Each experiment was carried out in triplicate. Values in the text are means \(\pm\) SEM \((n = 5-9)\).

Analyses were performed using the StatView 4.01 Non-FPU (Abacus Concepts, 1992–1993) statistical package.

Results

9-cis RA and all-trans RA stimulate PEPCK-C expression in 3T3–442A adipocytes. An 18-h treatment with 9-cis RA and all-trans RA resulted in both increased PEPCK-C protein and activity in 3T3-F442A adipocytes (Fig. 1A–C). Induction of PEPCK-C mRNA reached a maximum increase to \(~3\)-fold of the control at 4 h for 9-cis RA and all-trans RA (Fig. 2A). For both RA isomers, this increase was maintained at 24 h (data not shown). We chose a 4-h treatment to study the concentration-response relationship of RA action. Pck1 expression was more sensitive to 9-cis RA than to all-trans RA, because we observed with 10 nmol/L of 9-cis RA a significant change to 2-fold of the control and a maximal effect at 1 \(\mu\)mol/L, which represented an increase to 5-fold of the control (Fig. 2B). The concentration of 9-cis RA that produces half-maximum effect \((EC_{50})\) on PEPCK-C mRNA was \(\sim10\) nmol/L, while that of all-trans RA was \(\sim100\) nmol/L, as previously observed [20].

We chose a treatment time of 4 h and the maximal concentration of 1 \(\mu\)mol/L of both RA isomers to study the gene selectivity of effect. As expected from above, all-trans RA and 9-cis RA augmented PEPCK-C mRNA (Fig. 2B). In contrast, mRNA amounts for PEPCK-M, glycerol kinase (GyK), glycerol-3-P dehydrogenase (G3PDH), RXRa, and PPAR\(g\) were not significantly affected (results not shown). These results indicated that the RA effect was gene selective.

Differential mechanism of 9-cis RA and ATRA activation of Pck1 promoter. To characterize the DNA elements in the Pck1 promoter implicated in the response to 9-cis RA and all-trans RA, we used a series of plasmid constructs containing either the wild-type (WT) or mutated promoter of the rat Pck1 from –2086 to +69 bp relative to the transcription start site of the gene linked to the CAT gene that we transfected into 3T3-F442A adipocytes. Mutated bases were in the previously described elements RARE1/PCK1, RARE2, and RARE3/PCK2. We previously showed that mutations in RARE1/PCK1 and RARE2 partly abolished the response of the transfected gene to all-trans RA in hepatoma cells, whereas mutation in RARE3/PCK2 abolished TZD induction in adipocytes [11]. We decided to mutate RARE3/PCK2, because this element is able to bind a PPAR\(g\)/RXR heterodimer that is supposedly activated by both RXR and PPAR\(g\) ligands.

Transiently transfected WT construct 3T3-F442A adipocytes responded to an 18-h exposure to 9-cis RA and all-trans RA with a respective increase in CAT activity to 4.6- and 3.7-fold of the control (Fig. 3A). When adipocytes were transfected with the construct containing the triple mutated promoter in RARE1/PCK1, RARE2, and RARE3/PCK2, both inductions were completely abolished. These data suggest that the 3 elements carry the full response to both RA isomers and thus form the RA response unit of Pck1 promoter in 3T3-F442A adipocytes.

The role of the adipocyte-specific element RARE3/PCK2 was further studied using the Pck1 promoter mutated either in RARE3/PCK2 or in both RARE1/PCK1 and RARE2. RARE3/PCK2 was required for the full response to RA (Fig. 3B). Indeed, with the RARE3/PCK2-mutated construct, an 18-h exposure to 9-cis RA and all-trans RA increased CAT activity to only 1.9- and 2.3-fold of the control, respectively, thus representing diminished inductions of 63 and 38% compared with the response of the WT Pck1 promoter (compare Fig. 3A with B). Furthermore, the RARE1/PCK1-RARE2-mutated promoter (RARE3/PCK2 alone) was not activated when cells were exposed to all-trans RA. In contrast, this promoter was significantly activated by 9-cis RA with a change in CAT activity to 1.9-fold of the control. Taken together, these data suggest that RARE3/PCK2 is rather impli-
cated in the 9-cis RA effect than in the all-trans RA response. However, RARE3/PCK2 is required for the full response to both RA and remains an important cooperative element.

Using RARE3/PCK2-RARE1/PCK1 or RARE3/PCK2-RARE2-mutated promoters, we next evaluated the role of RARE1/PCK1 and RARE2 in the response of Pck1 promoter to RA. The RARE3/PCK2-RARE1/PCK1-mutated promoter (RARE2 alone) was significantly activated by 9-cis RA and all-trans RA (1.7- and 2.1-fold of the control, respectively) (Fig. 3C). However, the response of this construct was reduced by 63 and 44%, respectively, when compared with the WT promoter response (compare Fig. 3A with C). In contrast, the RARE3/PCK2-RARE2-mutated promoter (RARE1/PCK1 alone) was only increased by 9-cis RA to 2-fold of the control. This data indicated that RARE1/PCK1 was more specifically responsive to 9-cis RA, whereas RARE2 participated in both 9-cis RA and all-trans RA responses of Pck1 promoter, with a major involvement in all-trans RA activation.

9-cis RA and all-trans RA differentially regulate PEPCK-C in adipose tissue. To validate our results in a more physiological system, we cultured either subcutaneous WAT explants that we obtained from healthy overweight women or peri-epididymal WAT from Sprague-Dawley rats. Rat explants responded identically to a 24-h treatment with 1 μmol/L 9-cis RA or all-trans RA with a change to more than 10-fold of the control in PEPCK-C mRNA (not shown). In contrast, treatment of human explants with 1 μmol/L 9-cis RA caused a progressive increase in the abundance of the PEPCK-C transcript that reached a maximum to 3-fold of the control at 14 h and decreased thereafter (Table 1). Under the same conditions, all-trans RA did not modulate PEPCK-C mRNA (Table 1). As observed in 3T3-F442A cells, induction by 9-cis RA was gene selective since expression of PEPCK-M, GyK, and G3PDH remained unchanged at 14 h under 9-cis RA treatment (Fig. 4A).

The increase in PEPCK-C mRNA by 9-cis RA led to an activation of the glyceroenogenic flux since a 30% increase in [1-14C] pyruvate incorporation into neutral lipids was observed at 18 h of 9-cis RA treatment (Fig. 4B), whereas all-trans RA was inefficient and did not affect NEFA release (data not shown). As expected, activation of the glyceroenogenic flux and FA reesterification by 9-cis RA was concomitant with a 30% decrease in NEFA release from human WAT explants (Fig. 4B).

Discussion
In this report, we made a detailed analysis of 9-cis RA effect on Pck1 expression in the rodent adipocyte cell line 3T3-F442A in...
parallel to all-trans RA action on this gene. We show that both RA stereoisomers rapidly, strongly, and selectively induce PEPCK-C mRNA and protein. Both all-trans RA and 9-cis RA show a similar efficiency with a maximal effect attained following a 4-h treatment. However, sensitivity of the gene to 9-cis RA is much higher than to all-trans RA. The EC₅₀ for all-trans RA is quite similar to that previously demonstrated for expression of Pck1 in the same cells (20). This EC₅₀ is, however, relatively high when compared with the one determined for all-trans RA induction of Thrsrp (encoding S14) gene and the reduction of Retn encoding gene in 3T3-L1 adipocytes (25), suggesting the primary involvement of RXR compared with RAR in the effect of RA on these genes. Adipocytes have long been recognized as RA-responsive, because both RAR and RXR are expressed in such cells. Differences in sensitivity could reflect the differential involvement of these receptors. Both 9-cis RA and all-trans RA can directly bind to and activate RAR although with a much better affinity for all-trans RA, whereas RXR only binds 9-cis RA (26). These results are similar to those obtained in the liver in which Pck1 is a model for assessing retinoid regulation of liver-specific genes that selectively encode enzymes of carbohydrate metabolism (27). In hepatic cell lines, 9-cis RA produces a dose-dependent increase of fatty acid transport protein 1 and acyl-CoA synthase mRNA levels, both being also upregulated by all-trans RA but to a much lower extent than by 9-cis RA (28).

In liver cells as in adipocytes, all-trans RA exerts its inductive effect on Pck1 primarily at the transcriptional level (20). The mechanism of Pck1 induction by RA in rat hepatoma cells has been extensively characterized. The rat Pck1 contains 3 elements (RARE) in the 5’-flanking sequence of the gene: RARE1/PCK1, RARE2, and RARE3/PCK2 (12,13,19). When both RARE1/PCK1 and RARE2 were mutated and transfected into H4IIE rat hepatoma cells, 80% of all-trans RA response was lost (13). However, in those studies, mutated constructs started at −467 bp of the Pck1 promoter and RARE3/PCK2 at −997 bp was not considered.

We show here that RARE3/PCK2 is necessary for the full response of Pck1 to both all-trans RA and 9-cis RA in adipocytes. RARE3/PCK2 is also an adipocyte-specific element that binds a PPARY/RXRA heterodimer in a differentiation-dependent manner (29) and allows induction of Pck1 by TZD (11). In PPAR/RXR heterodimers, RXR is not silent, which means that RXR ligands can activate transcription through such PPAR-response elements (30). Alternatively, RARE3/PCK2 could bind a PPARY/δ/RXR heterodimer that all-trans RA could activate (18). All this is consistent with our observation that the integrity of RARE3/PCK2 is necessary for the full response of Pck1 to 9-cis RA and potentially to all-trans RA. Alternatively, the full RA response might be linked to the occurrence in adipocytes of a specific bridging between RARE3/PCK2 and the transcription preinitiation complex through an integrator or a coactivator like CBP/P300 or PGC-1, respectively, as suggested (29,31). Such bridging would ultimately interact with the other heterodimers bound to Pck1/RARE3/PCK2.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>5 h</th>
<th>14 h</th>
<th>24 h</th>
</tr>
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<tbody>
<tr>
<td>9-cis RA</td>
<td>100 ± 24.7</td>
<td>195.7 ± 19.5*</td>
<td>316.3 ± 69.1*</td>
<td>199.5 ± 82.1</td>
</tr>
<tr>
<td>All-trans RA</td>
<td>100 ± 24.7</td>
<td>139.5 ± 25.2</td>
<td>100.3 ± 23.1</td>
<td>125.8 ± 36.3</td>
</tr>
</tbody>
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1 Values are means ± SEM, n = 8. *Different from control, P < 0.05.

### Figure 4

9-cis RA regulates PEPCK-C and glyceroneogenesis in human adipose tissue. (A) Human subcutaneous WAT explants were treated or not with 1 μmol/L 9-cis RA for 14 h then PEPCK-C, PEPCK-M, GyK, and G3PDH mRNA levels were analyzed by real time RT-PCR. Results are normalized using GAPDH mRNA. (B) Glyceroneogenesis and NEFA release in the medium were monitored after an 18-h treatment with or without 1 μmol/L 9-cis RA. Data are means ± SEM, n = 8. *Different from control, P < 0.05.
The fact that 9-cis RA, but not all-trans RA, induces PKC expression in human WAT explants is somewhat surprising. However, such a phenomenon is not unique, because in the HepG2 human hepatoma cells, 9-cis RA induces expression of both fatty acid transport protein 1 and acyl-CoA synthase genes, whereas all-trans RA have no effect (28). This difference between rodent and human adipocytes could be linked to variations in expression or activity of one of the various heterodimeric partners (RXR/RAR) or one of the cofactors and bridging factors required for transcriptional activity in both cell types.

In conclusion, our results show unequivocally that 9-cis RA acts as a strong inducer of the PEPCCK-C gene in both humans and rodents, thereby leading to increased gluconeogenesis and eventually to reduced fatty acid efflux from adipocytes. This observation could be pathophysiological relevant, as we may now consider that rexinoids such as TZD could be used in situations of fatty acid-induced insulin resistance and type 2 diabetes.

**Literature Cited**

18. Schug TT, Berry DC, Shaw NS, Travis SN, Noy N. Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. Cell. 2007;129:723–33.

Retinoids and PEPCCK in adipose tissue